Refined Crystal Structures of *Escherichia coli* and Chicken Liver Dihydrofolate Reductase Containing Bound Trimethoprim

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Refined crystal structures are reported for complexes of *Escherichia coli* and chicken dihydrofolate reductase containing the antibiotic trimethoprim (TMP). Structural comparison of these two complexes reveals major geometrical differences in TMP binding that may be important in understanding the stereochemical basis of this inhibitor's selectivity for bacterial dihydrofolate reductases. For TMP bound to chicken dihydrofolate reductase we observe an altered binding geometry in which the 2,4-diaminopyrimidine occupies a position in closer proximity (by approximately 1 Å) to helix αB compared to the pyrimidine position for TMP or methotrexate bound to *E. coli* dihydrofolate reductase. One important consequence of this deeper insertion of the pyrimidine into the active site of chicken dihydrofolate reductase is the loss of a potential hydrogen bond that would otherwise form between the carbonyl oxygen of Val-115 and the inhibitor's 4-amino group. In addition, for TMP bound to *E. coli* dihydrofolate reductase, the inhibitor's benzyl side chain is positioned low in the active-site pocket pointing down toward the nicotinamide-binding site, whereas, in chicken dihydrofolate reductase, the benzyl group is accommodated in a side channel running upward and away from the cofactor. As a result, the torsion angles about the C5-C7 and C7-C1' bonds for TMP bound to the bacterial reductase (177°, 76°) differ significantly from the corresponding angles for TMP bound to chicken dihydrofolate reductase (−85°, 102°). Finally, when TMP binds to the chicken holoenzyme, the Tyr-31 side chain undergoes a large conformational change (average movement is 5.4 Å for all atoms beyond C8), rotating down into a new position where it hydrogen bonds via an intervening water molecule to the backbone carbonyl oxygen of Trp-24.

Hydrofolate to tetrahydrofolate. Since various tetrahydrofolate-derived cofactors are required to mediate the transfer of one-carbon fragments in the biosynthesis of amino acids, thymidylic acid, and purines, inhibition of dihydrofolate reductase and consequent depletion of the tetrahydrofolate pool results in cessation of cell growth. Dihydrofolate reductase inhibitors that are useful in clinical applications (the so called "antifolates" or folate antagonists) include the antibacterial TMP, the antimalarial pyrimethamine, and the antineoplastic agent MTX.

An interesting characteristic of the interaction between dihydrofolate reductase and its inhibitors is the high degree of species selectivity exhibited by some types of inhibitors. TMP, for instance, binds about 3000 times more strongly to the *Escherichia coli* enzyme than to the vertebrate enzyme (Baccanari et al., 1982). This selectivity is an important factor contributing to the attractiveness of dihydrofolate reductase as a target for chemotherapy, and further advances in the design of specific new antifolates could be expected if it were possible to account for it structurally. With this idea in mind, we have been applying x-ray-diffraction methods to study the structures of various species of dihydrofolate reductase and to characterize the stereochemistry of the interactions between these enzymes and their cofactors, substrates, and inhibitors.

This paper describes the refined 2.3-Å structure of the *E. coli* dihydrofolate reductase binary complex containing bound TMP and proposes a hypothetical model for the ternary complex containing both NADPH and TMP which can account for the observed cooperativity between inhibitor and cofactor binding (Baccanari et al., 1982). It should be noted that the *E. coli* dihydrofolate reductase discussed here was obtained from a bacterial strain designated RT500, whereas a different strain (MB1428) was the source of enzyme used in our recently reported high-resolution study of the *E. coli* dihydrofolate reductase-MTX complex (Bolin et al., 1982). On the basis of amino acid sequence studies (Stone et al., 1977; Baccanari et al., 1981; Bennett et al., 1978), the two strains of *E. coli* dihydrofolate reductase are thought to differ at three positions. However, as discussed below, our x-ray results suggest that some of these putative differences may not exist and that, except for the glutamate-to-lysine mutation at position 154, the two strains of dihydrofolate reductase otherwise have identical tertiary structures.

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** The abbreviations used are: TMP, trimethoprim; MTX, methotrexate; Wat, water.
We also report here the refined 2.0-Å structure of the chicken holoenzyme (which is representative of all the closely homologous vertebrate dihydrofolate reductases) and the refined 2.2-Å structure of chicken dihydrofolate reductase containing both NADPH and TMP. Comparison reveals that enzyme inhibitor interactions, as well as the conformations of the bound TMP molecules themselves, are distinctly different in the E. coli and chicken dihydrofolate reductase-TMP complexes. As will be detailed in the following paper (Matthews et al., 1985), these differences provide a structural explanation for the species selectivity of TMP. Preliminary structural results for the E. coli and chicken dihydrofolate reductase-TMP complexes were reported previously, but at lower resolution and without crystallographic refinement (Baker et al., 1981; Matthews and Volz, 1982).

**CRYSTALS, X-RAY DATA, AND STRUCTURE REFINEMENT**

*E. coli Dihydrofolate Reductase - Trimethoprim Binary Complex—* E. coli dihydrofolate reductase (form I, strain RT500) was crystallized as the binary complex with TMP from aqueous ethanol using a vapor diffusion method (Baker et al., 1981). The enzyme (20 mg/ml) was dissolved in 10% ethanol containing 0.5 mM TMP, 3.66 mM calcium chloride, and 50 mM histidine HCl at pH 6.8 and dialyzed against two changes of the same solution. Droplets of this enzyme solution were equilibrated in a vapor-diffusion apparatus with a reservoir containing 20% ethanol. Large hexagonal bipyramidal crystals (1.2 mm) grew in approximately 1 month. The crystals had space group P6₁, with unit cell parameters a = 93.3 A and c = 74.0 Å, which agree closely with those reported for the strain MB1428 E. coli dihydrofolate reductase binary complex with MTX (P6₆; a = 93.2 Å, c = 73.6 Å, Matthews et al., 1977).

Three-dimensional x-ray data were collected on an Arndt-Wonacott rotation camera. A single crystal was used to cover a total rotation range of 64° about the crystallographic c axis, thus achieving at least a 4-fold redundancy for all data with the exception of reflections close to the c axis, which were inaccessible in the rotation geometry described. All films were processed on an Optorics P1000 drum scanner linked to a PDP 11/45 computer. Approximately 62,000 intensity measurements were made to a maximum resolution of 2.35 Å, resulting in 12,825 unique observations representing 83% of the total possible data within the 2.35 Å measurement sphere. Data reduction included correction for absorption and resulted in an overall internal R_sym value for repeated measurements (∑ₙᵣₙ(∑ᵣ|T − Fₘₙ|/∑ₙᵣₙ) of 0.109.

A starting model for use in initiating crystallographic refinement was obtained as follows. The protein portion of the model was derived from the refined crystal structure (R = 0.155 at 2.5-Å resolution) of E. coli dihydrofolate reductase strain MS3746 (Poe et al., 1979), containing bound MTX.² Dihydrofolate reductase from strain MS3746 has glutamate at position 154 and is thus even closer in structure to RT500 dihydrofolate reductase than is the MB1428 enzyme. Tri- methoprim was placed in the substrate-binding site of this starting model by superimposing protein α-carbon coordinates with those of a second model consisting of the unrefined α-carbon and trimethoprim coordinates of the dihydrofolate reductase-TMP structure determined by Baker et al. (1981).

This procedure was performed independently for both molecules in the asymmetric unit on an Evans and Sutherland computer graphics terminal.

² D. A. Matthews, J. T. Bolin, and J. Kraut, unpublished results.

A total of seven cycles of restrained-parameter least-squares refinement (Hendrickson and Konnert, 1980) were calculated. The nonlinear F_calc to F_obs scaling function described by Bolin et al. (1982) was used throughout the refinement to allow for the contribution of liquid-solvent scattering to F_obs values for the parameters k₁ and B₂ were 0.83 and 420 Å², respectively. Difference Fourier maps were examined after the third, fifth, and seventh cycles.

The final model has an R value of 0.19 calculated over all measured data within the range 20 Å to 2.3 Å. Bond lengths are restrained to a root-mean-square deviation of 0.05 Å from their ideal values and the root-mean-square deviation of atoms in planar groups from their least-squares plane is 0.01 Å. These refinement statistics correspond to a mean uncertainty in atomic position of about 0.20 Å (method of Luzzati, 1952). An independent estimate of positional errors in well-ordered regions of the structure can be obtained by least-squares superposition of α-carbon coordinates for corresponding atoms in the two crystallographically independent molecules. If coordinates for 15 residues near the dimer interface are excluded from such a comparison (because of obvious conformational differences in the two molecules), the average difference between equivalent α-carbons is 0.25 Å. This average deviation is an upper bound for the true error since part of the discrepancy probably is real and occurs simply because equivalent atoms in the two dihydrofolate reductase molecules are in slightly different environments.

A total of 2537 protein and inhibitor atoms are contained in the final model. Forty of the expected protein atoms are absent from the model; all are in the side chains of exposed residues. The model also contains 191 water oxygen atoms, 2 chloride ions and a single calcium ion. The most significant refinement corrections from the starting model are: 1) the side-chain conformation of Arg-52, 2) the fixed-solvent structure near the side chains of Lys-32 and Arg-57, and 3) the fixed-solvent structure near the exposed edge of the inhibitor's pyrimidine ring. These will be discussed in later sections.

*Chicken Dihydrofolate Reductase Holoenzyme—* Crystals of chicken dihydrofolate reductase holoenzyme (the binary complex containing only bound cofactor) were grown as described previously (Volz et al., 1982). A single crystal of the holoenzyme was mounted on the Multiwire Area Detector Diffractometer (MADD) developed by Xuong et al. (1978) and used to collect 63,757 observations of 14,106 reflections to a Bragg spacing of 2.0 Å. Crystal decomposition at the end of the data collection was estimated to be 15% and the final value for R_sym was 0.054. This set of diffraction data plus protein and cofactor coordinates from the initial structure determination of the chicken dihydrofolate reductase ternary complex containing NADPH and methoxyphenyltriazine (Volz et al., 1982) served as a starting point for least-squares crystallographic refinement using the Hendrickson and Konnert (1980) program. The final refined model for the holoenzyme is the result of nine rounds of model reconstruction with least-squares refinement iterated to convergence after each reconstruction step. The holoenzyme model consists of 1854 protein atoms, 48 NADPH atoms, 281 fixed water molecules (with variable occupancy factors) and a single calcium ion. Overall isotropic thermal motion and low angle x-ray scattering from the liquid solvent are modeled (as described by Bolin et al., 1982) with k₂ = 0.734 and B₂ = 291 Å². The model for the holoenzyme gives an R factor of 0.22 with a 0.06-Å root-mean-square deviation of the bond lengths from ideality.

*Chicken Dihydrofolate Reductase-TMP-NADPH Ternary Complex—* The chicken dihydrofolate reductase ternary complex containing both NADPH and TMP was prepared by
soaking selected crystals of the holoenzyme for 1 week in a pH 6.8 artificial mother liquor containing 0.05 M sodium cacodylate, 0.005 M calcium acetate, 0.001 M NADPH, 40% ethanol, and a saturating concentration of TMP. Unit cell parameters and x-ray data collection procedures for the TMP ternary complex were the same as those described earlier for the holoenzyme (Volz et al., 1982). Three crystals were used to collect 13,799 observations of 11,362 unique reflections, representing a complete set of diffraction intensities to 2.2 Å resolution. Crystal decomposition was followed by periodically monitoring the intensities of four standard reflections, and data collection on each individual crystal was terminated when an intensity decrease of 10% was noted for any standard reflection. A larger set of 45 reflections was also scanned four or five times during data collection on each crystal in order to provide an expanded basis for intensity scaling. The final \( R_{\text{sym}} \) for all data was 0.033.

Structural changes upon binding TMP, including identification of the inhibitor itself and movements of various protein atoms, were initially deduced from difference electron-density maps computed from 2.8-Å data with the original multiple isomorphous replacement phases obtained for the triazine ternary complex (Vols et al., 1982).

The refined holoenzyme structure was taken as a starting model for refinement of the TMP ternary complex except for two adjustments: 1) one molecule of fixed solvent in the holoenzyme that overlapped the TMP-binding site was removed and replaced by TMP in an orientation determined by inspection of the initial difference map and 2) the side chain of Tyr-31 was repositioned to be consistent with features in the initial difference map that suggested a substantial conformational change for this residue upon binding TMP. It was also necessary to remove from the initial model of the TMP ternary complex two additional water molecules present in the holoenzyme structure since they would otherwise coincide with the new position of Tyr-31 in the ternary complex.

Structure factors were calculated with individual isotropic temperature factors and corrected for x-ray scattering from the liquid solvent by the method of Bolin et al. (1982), with

\[ k_0 \approx 0.75 \quad \text{and} \quad B_0 = 220 \quad \text{Å}^2. \]

The final model of the chicken dihydrofolate reductase ternary complex with NADPH and TMP is the result of eight rounds of model reconstruction with least-squares refinement iterated to convergence after each reconstruction step. This model gives an \( R \) factor of 0.21 for all data to 2.2 Å, and has a 0.01-Å root-mean-square deviation of bond lengths from ideal values. These refinement statistics correspond to a mean uncertainty in atomic position of about 0.20 Å (Luzzati, 1952).

**STRUCTURE OF THE E. COLI DIHYDROFOLATE REDUCTASE-TMP BINARY COMPLEX**

The trimethoprim-binding sites—TMP binds at the active site of *E. coli* dihydrofolate reductase with its pyrimidine ring occupying the interior extremity of a deep cleft, where it is held by a combination of van der Waals, hydrogen bonded, and ionic interactions with the protein. The trimethoxybenzyl side chain extends out toward the mouth of the binding cavity, making van der Waals contact with residues from aB and the carboxy terminal end of aC. As will be detailed later in this section, the geometry of TMP binding to *E. coli* dihydrofolate reductase closely resembles that of MTX binding, especially at their respective 2,4-diamino pyrimidine moieties, which interact identically with the enzyme. Structural formulas for TMP and MTX are compared in Fig. 1, and Fig. 2 shows TMP bound at the active site of the *E. coli* enzyme.

Residues located at the far interior end of the active site that are in van der Waals contact with the pyrimidine ring of TMP (Table I) are Ile-5, Ala-6, Ala-7, and Ile-94. Additional major hydrophobic contacts occur between the pyrimidine ring and the side chains of Phe-31 and Leu-28 located above the ring and adjacent to its exterior edge, respectively. The active-site region below the pyrimidine ring is open to solvent and contains several bound water molecules. Structural comparison with the *Lactobacillus casei* dihydrofolate reductase-MTX-NADPH ternary complex (Filman et al, 1982) suggests that, in the presence of bound cofactor, many of these fixed solvent molecules will be displaced in order to accommodate the bound nicotinamide ring (see below). As in the case of MTX binding (Bolin et al., 1982), the side chain of Asp-27 closely approaches both N1 and the 2-amino group of TMP in a geometry that is consistent with the existence of a hydrogen-bonded salt bridge. Evidence from NMR experiments indicates that the \( pK_a \) of TMP is increased from about 7.5 in solution to over 10 upon binding to dihydrofolate reductase (Roberts et al., 1981; Cocco et al., 1983), the effect presumably occurring at N1. Thus, N1 is probably protonated and Asp-27 deprotonated in the complex. A similar \( pK_a \) effect is seen in enzyme-bound MTX (Cocco et al., 1981, and references therein). A further point of similarity between the MTX and TMP complexes is that a tightly bound solvent molecule (Wat-639) mediates a second hydrogen bond between the 2-amino group and the side chain hydroxyl of the conserved residue Thr-113. Finally, to complete the comparison between the environment of the pyrimidine rings in bound MTX and TMP, the same pair of backbone carbonyl oxygens (Ile-5 and Ile-94) lie in the plane of the pyrimidine ring, positioned to accept hydrogen bonds from the 4-amino group. The striking resemblance between the binding stereochemistry of these two inhibitors will be further discussed below.

The crystallographic evidence suggests that an important factor underlying the strong affinity of TMP for *E. coli* dihydrofolate reductase is the favorable van der Waals interaction between the trimethoxybenzyl group of TMP and protein residues on two helices, aB and aC, near the entrance to the active site. Ser-49 and Ile-50 are located at the C-terminal end of aC where they form a wall, composed of both main-chain and side-chain atoms, against which the trimethoxybenzene ring of TMP rests. An additional van der Waals contact on the left side of the binding cavity (as seen in Fig. 2) occurs between C82 of Leu-54 and one of the inhibitor's meta-methoxy groups. On the opposite side of the active site cleft, Leu-28, in the middle of aB, provides the major protein

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**Fig. 1.** Covalent structure and atom numbering for methotrexate (top) and trimethoprim.
Fig. 2. The binding of the trimethoprim to E. coli dihydrofolate reductase. Trimethoprim is indicated by solid bonds and protein by open bonds. Carbon atoms are represented by smaller open circles, oxygen atoms by larger open circles, and nitrogen atoms by blackened circles. Large numbered circles represent fixed solvent molecules. Hydrogen bonds, indicated by dashed lines, are listed in Table I.

Table I

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<th>Component of TMP</th>
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<th>Contact chicken dihydrofolate reductase</th>
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<td>Ala-9 N, Ca, Cβ</td>
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<tr>
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*ec indicate the E. coli enzyme.

Contacts with the side chain of TMP. The side chain of Leu-28 is adjacent to the upper half of TMP's trimethoxybenzene ring, closely approaching C2', C3', C4' and their attached groups while the lower portion of this face of TMP is exposed to solvent. A second residue in αB, namely Phe-31, is positioned 3.9 Å from the carbon atom of the meta-methoxy group, shown extending upwards in Fig. 2.

Although all three methoxy groups are bound near the entrance to the active-site cleft, their accessibilities to solvent are distinctly different. The upper meta-methoxy group is
effectively sequestered from solvent except for the proximity of its methyl group to Wat-773 and Wat-774, both about 4 Å away. The para-methoxy oxygen atom in molecule II is 3.3 Å from Wat-670 but only 3.0 Å from Wat-570. The geometrical arrangement of Wat-570 and the para-methoxy oxygen is consistent with the existence of a hydrogen-bonded interaction between the two. Although well-documented hydrogen bonds involving methoxy groups are rare, they are not unknown (Domak and Riche, 1977). The remaining meta-methoxy group, by far the most solvent-exposed of the three, is surrounded by a constellation of fixed water molecules. However, it should be borne in mind that the structure we are describing here is the TMP binary complex, from which NADPH is absent, and that the environment of this meta-methoxy group is undoubtedly different in the biologically more relevant ternary complex containing NADPH. Later we will consider possible structural differences between the binary and ternary complexes of E. coli dihydrofolate reductase.

Conformation of Bound Trimethoprim—The trimethoprim molecule has two principal degrees of conformational freedom, namely torsional rotations around C(5)-C(7) and C(7)-C(1'). These are designated $\theta_1$ and $\theta_2$, respectively. In addition, the three methoxy groups can adopt in-plane or out-of-plane conformations, with torsion angles designated $\tau_1$, $\tau_2$, and $\tau_3$.

A model for TMP binding to L. casei dihydrofolate reductase in solution has already been proposed by Cayley et al. (1979) based on nuclear magnetic resonance transfer-of-saturation experiments which permitted identification of proton resonances in the bound inhibitor. From our crystallographic structure of the TMP binary complex with E. coli dihydrofolate reductase, we find $\theta_1 = 177^\circ$ and $\theta_2 = 76^\circ$, resulting in a conformation that positions the two aromatic rings nearly perpendicular to one another. This conformation closely resembles one of two predicted from the magnetic-resonance studies just cited. A similar geometry has been found for the free molecule in crystals of various salts of TMP and in certain close structural analogs (Phillips and Bryan, 1969; Giuseppetti and Tadini, 1980; Cody, 1984). However, for TMP itself and for dimeridine (5'-desmethoxy TMP) the corresponding values for $\theta_1$, $\theta_2$ are $-89^\circ$, $153^\circ$ (Koetzle and Williams, 1976) and $-66^\circ$, $153^\circ$ (Koetzle and Williams, 1978), respectively. Thus, we are reminded once again that the conformation of a small molecule in the crystalline state may differ from its conformation when bound to a macromolecular receptor. The interesting question of whether or not variations in $\theta_1$ and $\theta_2$ (and, therefore, variations in conformational energy) are important in explaining TMP's selectivity for certain dihydrofolate reductases is considered in the following paper (Matthews et al., 1985).

Turning our attention to the methoxy group torsion angles, we find that in molecule II all three substituents are nearly coplanar with the benzene ring of TMP ($\tau_1 = 22^\circ$, $\tau_2 = 13^\circ$, $\tau_3 = 153^\circ$). In molecule I, however, the para-methoxy is rotated out of the plane ($\tau_2 = -65^\circ$). The presence of Wat-570 in the binding site of molecule II hydrogen bonds to the para-methoxy oxygen and the absence of a corresponding molecule of bound solvent in molecule I may well be the most significant factor in accounting for the $\tau_2$ torsion-angle differences when TMP binds to crystallographically independent dihydrofolate reductase molecules. In molecule II, Wat-570 is linked via intermolecular hydrogen bonds to the backbone carbonyl of Ala-145(I) and N2 of Gln-146(I). The close proximity of this water to one face of TMP's trimethoxybenzyl side chain would push these conformations with $\tau_2$ significantly out of plane. Since the occurrence of Wat-570 in molecule II apparently depends on its interactions with molecule I across the dimer interface, its presence is most probably an artifact of crystallization, and it is likely that in solution the lower energy out-of-plane torsion angle observed for the para-methoxy group in molecule I will be favored.

Similarity of TMP and MTX Binding—Within our ability to distinguish at this resolution, as we have already noted above, the 2,4-diaminopyrimidine rings of TMP and MTX are bound identically in their respective binary complexes with E. coli dihydrofolate reductase (Figs. 2 and 3). Moreover, comparison of the interactions made by the enzyme with the rest of each inhibitor molecule shows that the similarity extends beyond their pyrimidine rings. Owing to the 177° torsion angle for $\theta_1$, C7 and C1' of TMP occupy positions closely analogous to those occupied by N5 and C6 in enzyme-bound MTX. Additionally, when the protein portions of the two binary complexes are superimposed, the benzene side chains of TMP and MTX are coplanar and partially overlapping, even though they are separated from their individual pyrimidine rings by two and five bonds, respectively, both benzene rings make contact with side chains of Leu-28 and Ile-50. Further comparison of the superimposed binary complexes reveals that the positions of C3', C4', and C5' of TMP correspond (within 0.4 Å) to those of C2', C1', and N10, respectively, in bound MTX, while the methoxy oxygen atoms O3', O4', and O5' are each within 1 Å of C3', C6', and C11 of MTX. Thus, a notable conclusion from this analysis is that, despite their evident structural differences, the bound side chains of TMP and MTX are stabilized by generally very similar van der Waals contacts with E. coli dihydrofolate reductase.

With the single exception of Arg-52, there are no significant ligand-induced protein conformational differences between the dihydrofolate reductase-TMP and dihydrofolate reductase-MTX binary complexes. The side chain of Arg-52, in the case of the E. coli dihydrofolate reductase-MTX binary complex, is almost completely extended, occupying a position on the left side of the binding site where it can hydrogen bond directly to the benzoyl carbonyl oxygen of MTX and indirectly to the same carbonyl oxygen via an intervening fixed solvent molecule (Bolin et al., 1982). The absence of a similarly disposed hydrogen bond acceptor group in TMP causes a repositioning of the Arg-52 side chain (in molecule II) to a location where it can instead hydrogen bond to the backbone carbonyl of Leu-54. However, in molecule I of the same binary complex no significant electron density appears for any side-chain atom of Arg-52 beyond C6, indicating that in this crystallographically independent molecule the guanidinium group of Arg-52 is disordered.

A MODEL FOR THE TERNARY COMPLEX E. COLI DIHYDROFOLATE REDUCTASE-TMP-NADPH; LIGAND COOPERATIVITY

Baccannari et al. (1982) have shown that the affinity of TMP for E. coli holo-dihydrofolate reductase (containing cofactor) is about 3000 times greater than for mouse lymphoma holo-dihydrofolate reductase. Such species selectivity is one of the important factors underlying TMP's value as an antibacterial agent. It is particularly notable, however, that this selectivity depends on enhanced cooperativity between NADPH binding and TMP binding to the E. coli enzyme, in
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FIG. 3. The binding of methotrexate to E. coli dihydrofolate reductase. Methotrexate is indicated by solid bonds and protein by open bonds. Carbon atoms are represented by smaller open circles, oxygen atoms by larger open circles, and nitrogen atoms by blackened circles. Fixed solvent molecules have been omitted. Hydrogen bonds between the pyrimidine portion of methotrexate and the protein are indicated by dashed lines.

contrast to a much less marked cooperativity exhibited by the vertebrate enzyme. Specifically, Baccannari et al. (1982) have shown that the E. coli dihydrofolate reductase dissociation constant for trimethoprim is decreased 40-fold in the presence of NADPH, whereas, for mouse lymphoma dihydrofolate reductase, the corresponding decrease is only a factor of 2.8. In other words, the selectivity of TMP for E. coli versus vertebrate dihydrofolate reductase is more than 14 times as great in the case of the holoenzyme as it is in the case of the apoenzyme. It is relevant, therefore, to inquire into the stereochemistry of NADPH binding to the E. coli dihydrofolate reductase-TMP binary complex, anticipating that such an analysis might lead to a better understanding of the mechanism underlying this cooperativity. Unfortunately, we have not yet been able to determine directly the crystal structure of any E. coli dihydrofolate reductase-inhibitor complex that also contains bound cofactor, although such work is now in progress at Wellcome Laboratories in Beckenham. We have, however, obtained refined high-resolution crystal structures for NADPH-containing complexes of both L. casei and chicken dihydrofolate reductase (Bolin et al., 1982; Volz et al., 1982) which permit us to predict, with considerable confidence, how NADPH would probably bind to the E. coli enzyme.

For this purpose, hypothetical coordinates for NADPH bound to E. coli dihydrofolate reductase were derived by superimposing the refined high-resolution structure of the L. casei dihydrofolate reductase-MTX-NADPH ternary complex onto that of the E. coli dihydrofolate reductase-MTX binary complex while simultaneously optimizing overlap of the two highly homologous enzyme-MTX portions of the two structures. The important result from this procedure is that an NADPH molecule is evidently able to bind to E. coli dihydrofolate reductase in almost precisely the same way as it does to L. casei dihydrofolate reductase, making a very similar set of cofactor-enzyme interactions. One notable difference involves the side chain of Met-20 in the E. coli enzyme, which is equivalent to Leu-19 in L. casei (and also leucine in all other known dihydrofolate reductase sequences). The role of Met-20 in cofactor binding will be discussed shortly.

To arrive at a hypothetical model for the E. coli dihydrofolate reductase-TMP-NADPH complex we then simply assume that TMP can be substituted for MTX without disturbing the way in which NADPH binds. This assumption is plausible since we already know† that it is true for a variety of other heterocyclic inhibitors bound to chicken dihydrofolate reductase.

The resulting model, depicted in Fig. 4, suggest that enhanced binding of TMP to E. coli dihydrofolate reductase in the presence of NADPH could be due to one or more of the following factors: 1) direct van der Waals interactions between the benzyl ring of TMP and the nicotinamide mononucleotide portion of NADPH, 2) increased desolvation of TMP itself, and/or 3) induced enzyme conformational changes at Met-20 that create new enzyme-TMP contacts and further desolate the inhibitor. These three possibilities will now be considered in turn.

It is apparent from Fig. 4 that, in the hypothetical ternary complex, the nicotinamide mononucleotide portion of NADPH is located very near the trimethoxybenzyl side chain of TMP. In particular, a total of seven nonbonded contacts of less than 5.0 Å occur between the nicotinamide or its associated ribose ring and C1', C6', or C10 of TMP. It appears from our model that TMP's principal torsion angles about the benzyl carbon would be unaffected by cofactor binding in agreement with NMR results (Cayley et al., 1979; and references therein).

Another important consequence of this proximity between cofactor and inhibitor is that, upon binding to dihydrofolate reductase, the NADPH molecule must expel a significant fraction of the bound solvent located in the lower portion of the active site. Our modeling experiment suggests that, in the immediate vicinity of bound TMP, at least seven water molecules, and possibly nine, will be displaced by the coenzyme. Thus, cofactor-induced desolvation of the inhibitor could

† D. A. Matthews, and K. W. Volz, unpublished results.
enhance binding of TMP to the holoenzyme relative to the apoenzyme.

Finally, an independent line of evidence prompts us to suggest that, upon binding NADPH, the side chain of Met-20 may undergo a conformational change that repositions it next to the trimethoxybenzyl group of TMP, permitting favorable noncovalent interactions between the two and also further inducing desolvation of the trimethoxybenzene ring. The principal evidence supporting this idea comes from crystallographic studies of L. casei and chicken dihydrofolate reductase ternary complexes containing NADPH plus inhibitors of various types. In both these cases, the leucine side chain corresponding to Met-20 is directed "up" toward the TMP molecule, whereas, in the binary complex of E. coli dihydrofolate reductase (not containing NADPH), the side chain of Met-20 is positioned well below any portion of the bound ligand. Of course we cannot dismiss the alternative possibility that these observed structural differences between the E. coli and the other species of dihydrofolate reductase may be unrelated to cofactor binding; for example they may simply be due to intrinsic structural properties of the enzyme molecules, or the conformation of the loop connecting βA to αB (containing Met-20) may be perturbed by crystal packing forces in the E. coli dihydrofolate reductase complexes (see Bolin et al., 1982). Additional evidence pertaining to these various possibilities will be forthcoming when x-ray studies now in progress (at Wellcome in Beckenham) on the E. coli dihydrofolate reductase-NADPH-TMP ternary complex are completed.

STRUCTURAL DIFFERENCES BETWEEN TWO STRAINS OF E. COLI DIHYDROFOLATE REDUCTASE

Dihydrofolate reductase has been isolated and purified from several different strains of E. coli bacteria. We have already emphasized that the enzyme composing the dihydrofolate reductase-MTX binary complex structure was derived from the E. coli strain designated MB1428, whereas that of the dihydrofolate reductase-TMP binary complex structure came from the strain RT500. It is, therefore, important to establish that no other significant structural differences exist between these two strains of E. coli dihydrofolate reductase.

According to published amino acid sequences, dihydrofolate reductase from E. coli strains RT500 (form I) and MB1428 differ in three positions out of 159 (Stone et al., 1977; Bennett et al., 1978; Baccanari et al., 1982). Residues 118, 142, and 154 are reported to be glutamine, aspartate, and glutamate in the RT500 enzyme while sequence data suggest that glutamate, asparagine, and lysine occupy analogous locations in MB1428 dihydrofolate reductase. Two of these putative differences involve conversion of an acidic residue into its corresponding amide. In view of the frequency with which sequencing artifacts of this type occur, and since we now possess refined crystallographic models for the dihydrofolate reductase from both strains, it is relevant to ask whether any of these putative sequence differences can be confirmed or discarded on purely structural evidence. Although the x-ray structure cannot directly distinguish between carboxylate and carboxamide groups, under favorable circumstances it is possible to infer the correct choice from hydrogen-bonding considerations.

In one instance, residue 142, we can in fact make an unambiguous judgment. According to Bennett et al. (1978), residue 142 in the MB1428 enzyme is asparagine, in contrast to the RT500 sequence (Stone et al., 1977) which has an aspartic acid at the corresponding position. However, the structure reveals that, in the MB1428 E. coli dihydrofolate reductase-MTX binary complex, the side chain of residue 142 in molecule II makes a pair of hydrogen bonds with the guanidinium group of invariant Arg-159 in a symmetry-related molecule. The geometry is nearly indistinguishable from that observed for the hydrogen-bond-mediated ion-pair interaction between the a-carboxylate of bound MTX and the guanidinium group of invariant Arg-57 (Bolin et al., 1982). Thus, the

\[ ^2 \text{Mapping of the structural gene for dihydrofolate reductase from E. coli K12 (wild type) indicates a primary sequence the same as that for strain RT500 (form I) except for the following changes: 37, aspartate → asparagine; 57, asparagine → aspartate; 118, glutamine → glutamate (Smith and Calvo, 1980).} \]
crystallographic evidence clearly favors the conclusion that residue 142 is aspartic acid and not asparagine in both strains of E. coli dihydrofolate reductase. However, we cannot of course rule out the possibility that deamidation of residue 142 took place during handling and crystallization of the MB1428 enzyme.

Dihydrofolate reductase from strain MB1428 has a lysine at position 154. Its side-chain nitrogen is hydrogen bonded to the hydroxyl oxygen of Ser-138 in the same molecule. In the RT500 enzyme, residue 154 is a glutamate which hydrogen bonds to both the hydroxyl side chain of Ser-135 and to an imidazole nitrogen of His-114. One apparent consequence of this lysine-to-glutamate mutation is that the side chain of His-114 is positioned slightly differently (∼0.8 Å) in the two dihydrofolate reductases.

**The Chicken Dihydrofolate Reductase-TMP-NADPH Ternary Complex**

The Trimethoprim-binding Site—TMP occupies the same site on the surface of chicken dihydrofolate reductase as does the phenyltriazine-inhibitor molecule described in our previous publication (Vollz et al., 1982). Both the pyrimidine ring of TMP and the triazine ring of the phenyltriazine occupy a cleft formed by the amino-terminal β strand, βA, and the helix following it, αB, while the trimethoxybenzyl side chain of TMP fills the same hydrophobic pocket as does the methoxyphenyl ring of the phenyltriazine inhibitor. Also, as would be expected, the pyrimidine- and triazine-binding site in chicken dihydrofolate reductase is analogous to the site in the bacterial dihydrofolate reductases (Bolin et al., 1982) that binds the pteridine end of a MTX molecule. Fig. 5 depicts the bound TMP molecule and its immediate surroundings in chicken dihydrofolate reductase.

Despite these resemblances, however, an important finding of the present study is that there are certain unexpected peculiarities about the way TMP binds to chicken dihydrofolate reductase. One such observation is that the pyrimidine ring is inserted almost 1 Å more deeply into the active-site cleft than are the heterocycles of either the phenyltriazine bound to the chicken enzyme, on the one hand, or of TMP or MTX bound to the bacterial enzyme on the other hand. Moreover, comparison of Figs. 2 and 5 immediately reveals that TMP binds to the chicken and E. coli enzymes in distinctly different conformations. These comparisons will be examined more deeply below, and their significance for understanding the species selectivity of various dihydrofolate reductase inhibitors will be discussed in the following paper (Matthews et al., 1985). But first, we briefly describe the effects TMP binding has on the holoenzyme structure of chicken dihydrofolate reductase, including those due to perturbations of protein-bound solvent.

**Effect of TMP Binding on the Holoenzyme Structure**—Since crystals of the chicken holoenzyme are isomorphous with those of the dihydrofolate reductase-TMP-NADPH ternary complex, these two structures can be compared by difference Fourier methods as well as by direct comparison of refined atomic coordinates. The difference Fourier method is especially well suited to discovering subtle structural perturbations due to TMP binding. Fig. 6 shows the substrate-binding site region of a difference map calculated at 2.2 Å resolution, on a 0.75 Å grid, using coefficients (F<sub>ternary</sub> - F<sub>holo</sub>)<sup>α</sup>, where α is the refined phase for the chicken holoenzyme complex. The most prominent difference-density feature corresponds to TMP itself, with a maximum peak height 9σ above background. Other significant features in the map indicate that when TMP binds to chicken dihydrofolate reductase, small movements of less than 1 Å occur involving the side chain of Gly-30 and both side-chain and main-chain atoms at the carbonyl end of αC. In addition, a much larger conformational change (∼5 Å) occurs at the side chain of Tyr-31. These structural adjustments will be considered more thoroughly later, following a discussion of TMP's effect on the displacement and rearrangement of bound solvent.

**Effect of TMP Binding on Solvent Structure**—Significant changes in solvent structure occur when TMP binds to the chicken holoenzyme. A total of three fixed water molecules are displaced, one of which (Wat-229) is lost because its atomic position overlaps the inhibitor-binding site, while two others (Wat-230 and Wat-427) are ejected as a result of the ligand-induced conformational change involving the side chain of Tyr-31. All three of the displaced waters are hydrogen bonded directly to polypeptide backbone and/or side chain atoms in the holoenzyme, with occupancies near unity. Among these highly ordered fixed solvent molecules, the loss of Wat-229 is especially noticeable in the difference map. Since its location in the holoenzyme is nearly identical with that for

![Fig. 5. The binding of trimethoprim and NADPH to chicken dihydrofolate reductase.](image-url)
the 2-amino group of TMP in the ternary complex, the computed difference density in the neighborhood of the 2-amino substituent is close to zero.

Comparison of solvent structure in the holoenzyme and ternary complexes also reveals that two of the remaining ordered active-site waters (Wat-335 and Wat-424) are positioned identically in both complexes but have increased occupancy in the presence of TMP. Finally, Wat-780 is also significantly more ordered in the ternary complex but, in addition, differs in position by about 1 Å from its location in the holoenzyme.

Comparison of Trimethoprim and Phenyltriazine Binding—A more detailed description of TMP binding in the chicken dihydrofolate reductase ternary complex is given in the next section, along with a structural comparison between this complex and the E. coli dihydrofolate reductase-TMP binary complex. In what follows here, we briefly consider TMP binding to chicken dihydrofolate reductase in relation to our earlier findings for phenyltriazine binding to the same enzyme (Volz et al., 1982).

Although the heterocyclic rings of TMP and phenyltriazine bind in the same active-site cleft of chicken dihydrofolate reductase, their precise fit into this pocket is different. Especially notable is the slight (1 Å) displacement of the pyrimidine ring of TMP across strand βA (from left to right as seen in Fig. 5) which increases the distance between the pyrimidine ring and βE while simultaneously moving the TMP molecule closer to βF and deeper into the pyrimidine-binding pocket. An important consequence of the altered pyrimidine-binding geometry characteristic of TMP in chicken dihydrofolate reductase is that the distance between the 4-amino group of the inhibitor and the backbone carbonyl oxygen of Val-115 is now 4.1 Å. Thus, in contrast to the chicken dihydrofolate reductase-phenyltriazine complex where a normal hydrogen bond exists between the triazine’s 4-amino substituent and the backbone carbonyl of Val-115, corresponding atoms in the chicken enzyme-TMP complex are too far apart for normal hydrogen bonding. Another noteworthy observation is that TMP and the phenyltriazine bind to chicken dihydrofolate reductase with their heterocyclic rings at slightly differing (25°) tilt angles. Except as noted above, the heterocyclic portions of TMP and phenyltriazine have qualitatively similar hydrophobic and polar contacts with chicken dihydrofolate reductase, including the important hydrogen-bond-mediated salt linkage between the active-site carboxylate of Glu-30 and the respective N1 and 2-amino groups.

The trimethoxybenzene ring of TMP binds to chicken dihydrofolate reductase in the same hydrophobic pocket as the methoxyphenyl group of the triazine inhibitor, although the two side chains are oriented somewhat differently. When models of the respective ternary complexes are superimposed, it is apparent that the benzene rings of the phenyltriazine and of TMP are in fact nearly perpendicular to one another.

Finally, we note that TMP and phenyltriazine, when bound to chicken dihydrofolate reductase, exert different effects on the conformation of Tyr-31. Whereas phenyltriazine binding produces only a minor side-chain adjustment (less than 1 Å) of Tyr-31 so as to enhance its van der Waals contact with the inhibitor’s methyl and phenyl substituents, TMP binding to the chicken holoenzyme is accompanied by a much larger conformational change.

Comparison of Trimethoprim Binding to Chicken and E. coli Dihydrofolate Reductases—Structural comparison of chicken and E. coli dihydrofolate reductase complexes containing TMP reveals several notable differences in inhibitor-binding geometry. The most obvious differences involve 1) hydrogen bonding at the 4-amino substituent of the pyrimidine ring, 2) enzyme-inhibitor interactions at the trimethoxybenzyl group, 3) inhibitor side-chain torsion angles, and 4) a major conformation change at the side chain of Tyr-31 when TMP binds to the chicken enzyme. These and other aspects of the comparative stereochemistry of TMP binding will now be examined in turn.

As discussed above, comparison of methoxyphenyltriazine and TMP bound to chicken dihydrofolate reductase reveals a small but observable difference in the placement of the respective 2,4-diaminoheterocycles. A similar displacement is observed when comparing the position of the bound pyrimidine rings of TMP in chicken and E. coli dihydrofolate reductases. Thus, whereas the 4-amino group of TMP participates in two hydrogen bonds with backbone carbonyl oxygens of E. coli dihydrofolate reductase, only one of the corresponding hydrogen bonds remains intact in the chicken dihydrofolate reductase-NADPH-TMP ternary complex—that between the 4-amino group of TMP and the backbone carbonyl of Ile-7.
The hydrogen-bond-mediated salt linkage between the active-site carboxylate of Glu-30 in chicken dihydrofolate reductase and TMP's N1 and 2-amino group is analogous to a similar geometrical arrangement involving Asp-27 and the pyrimidine of TMP bound to *E. coli* dihydrofolate reductase. In the holoenzyme complex, the carboxylate group of Glu-30 is apparently more mobile since a $2F_o - F$ map shows only very weak electron density beyond C'y. When TMP binds at the active site, Glu-30 is locked into position by its interaction with the inhibitor's N1 and 2-amino group. Evidence for reduced thermal motion and, perhaps, for a slight repositioning of the carboxylate as well can be seen in Fig. 6 as positive difference density coincident with the new position for the side chain of Glu-30. The 2-amino group also makes a second hydrogen bond to Wat-335, which, in turn, donates a hydrogen bond to the side-chain oxygen of Thr-136, a strictly conserved residue. A closely analogous hydrogen-bonding arrangement has been observed for other 2,4-diaminoheterocyclic inhibitors bound to both chicken and *E. coli* dihydrofolate reductases (Matthews et al., 1983; Kuyper et al., 1982; Baker et al., 1983).

Recent NMR data is also consistent with the notion that, at least for TMP and MTX, interaction between the inhibitor's protonated N1 and the active-site carboxylate group is very similar for dihydrofolate reductases from three bacterial and two vertebrate species (Cocco et al., 1983; Birdsell et al., 1983).

In chicken dihydrofolate reductase, most of the remaining protein contacts with the pyrimidine ring of TMP involve residues that are structurally analogous to residues performing a similar function in the *E. coli* dihydrofolate reductase-TMP binary complex (Table I). Note however that the 2 residues in chicken dihydrofolate reductase corresponding to Ile-94 and Leu-28 in *E. coli* dihydrofolate reductase, namely Val-115 and Tyr-31, are now more than 4 Å from the bound pyrimidine. In the case of Val-115, this increased separation is a direct result of the slight shift of the pyrimidine ring of TMP in the chicken dihydrofolate reductase complex.

It is apparent that protein interactions with the trimethoxybenzyl side chain of TMP are quite different in bacterial and vertebrate dihydrofolate reductases. In chicken dihydrofolate reductase the inhibitor's side chain has swung into an "up" conformation where it is accommodated in the same hydrophobic pocket that holds the side chains of various phenyltriazine inhibitors (Vozl et al., 1982; Matthews and Vozl, 1982). Five hydrophobic residues (Phe-94, Met-52, Ile-60, Leu-67, and Val-115) and the side-chain methyl of Thr-56 make van der Waals contacts with the trimethoxybenzyl side chain of TMP, while the side-chain oxygen of Gln-35 is positioned 4.0 Å from the inhibitor's *para*-methoxy group. Although the nicotinamide ring of NADPH occupies the lower portion of this cleft, van der Waals interactions between the nicotinamide ring and the inhibitor's trimethoxybenzene side chain must be weak since, in no instance, is the contact less than 4.0 Å (C7-nicotinamide to C7-TMP is 4.1 Å). The inhibitor's trimethoxybenzyl group is further shielded from solvent by the side chains of Leu-22 and Tyr-31, although interactions between these residues and TMP must also be weak as judged by separation distances which are almost all greater than 4.0 Å.

It is also clear from examination of the difference map (Fig. 6) that TMP binding to the chicken holoenzyme causes small structural perturbations of aC, particularly where this helix contacts the inhibitor's trimethoxybenzyl group. Negative difference density features ($3\text{e}F_o - F$) adjacent to residues 56-61, are proximal to bound TMP and are associated with corresponding positive features located nearby. Apparently, the carboxy end of helix aC moves about 0.1 to 0.2 Å in order to accommodate inhibitor binding. This movement probably also accounts for most of the difference density at and around the side chains of Ser-57 and Ile-60. However, the negative features are somewhat weaker than corresponding positive features, suggesting that, in this case, TMP binding may also reduce the apparent atomic thermal motion.

Recall that, for TMP bound to *E. coli* dihydrofolate reductase, two of the side-chain methoxy groups are positioned at the opening of the active-site cleft where they are extensively solvated by fixed water molecules. This can be contrasted with the much more lipophilic environment surrounding the analogous portion of TMP bound to the chicken enzyme.

It is important at this juncture to emphasize that differences in solvent accessibility to the bound side chains of TMP in the *E. coli* and chicken dihydrofolate reductase complexes are mainly attributable to the distinctly different manner in which the inhibitor binds to these two enzymes. Contrary to the impression that might be conveyed by a cursory examination of Figs. 2 and 5, the substrate-binding site of *E. coli* dihydrofolate reductase is not more highly solvated than that of chicken dihydrofolate reductase. The apparent difference in solvent accessibility is caused by the presence of cofactor in the chicken dihydrofolate reductase ternary complex, altered positioning of the side chain of Leu-22 relative to the structurally analogous residue in *E. coli* dihydrofolate reductase, namely Met-20 (which may also be related to the presence of cofactor in the chicken enzyme complex; see above), and net displacement of ordered solvent molecules in the chicken enzyme caused by inhibitor-induced movement of Tyr-31.

We have already seen that, in TMP bound to *E. coli* and chicken dihydrofolate reductase, the respective pyrimidine rings occupy closely analogous binding sites, in contrast to the corresponding trimethoxybenzyl groups which are accommodated in two distinctly different binding pockets. One consequence of these alternative binding modes is that the principal torsion angles about the C5-C7 and C7-C1' bonds of TMP are also different in the two dihydrofolate reductase-TMP complexes. In the *E. coli* reductase complex, the benzene and pyrimidine rings of TMP lie side by side ($\theta_1 = 177^\circ$, $\theta_2 = 76^\circ$) in contrast to the more open conformation for TMP bound to chicken dihydrofolate reductase ($\theta_1 = -85^\circ$, $\theta_2 = 102^\circ$), in which the inhibitor's benzyl side chain is positioned well above the plane of the pyrimidine ring.

Very recently, Birdsell et al. (1983) have attempted to derive the conformation for TMP bound to mouse L1210 dihydrofolate reductase by first determining chemical shifts for specific protons of bound TMP and then computing their expected shifts when $\theta_1$ and $\theta_2$ are systematically varied. The "most probable conformation" determined in this way has $\theta_1$ and $\theta_2$ equal to $-142^\circ$ and $100^\circ$, respectively. This value for $\theta_2$ agrees well with our values, namely $\theta_1 = -85^\circ$, $\theta_2 = 102^\circ$ for the chicken enzyme, but the value for $\theta_1$ is significantly different.

It is possible that this putative difference is real and that TMP binds to chicken and mouse dihydrofolate reductases in somewhat dissimilar conformations. However, we believe this to be extremely unlikely. The high amino acid sequence homology (75%-95%) among vertebrate dihydrofolate reductases (Vozl et al., 1982) suggests that their three-dimensional structures are nearly identical. Recently, the x-ray structure of mouse L1210 dihydrofolate reductase has been determined at 2.5-Å resolution and a preliminary comparison between it and chicken dihydrofolate reductase further supports this suggested similarity (Stammers et al., 1983).
A more likely explanation for the discrepancy in $\theta$, is that the hypotetical geometrical model of the mouse dihydrofolate reductase-TMP complex on which the chemical shift calculations were based is in error. The key assumption in these calculations is that the pyrimidine ring of bound TMP has the same spatial relationship to residues in the active site of mouse dihydrofolate reductase as does the pyrimidine portion of bound MTX to analogous residues in the L. casei enzyme. But we know from the x-ray results reported here (see below) that, when TMP binds to chicken dihydrofolate reductase (and therefore probably to mouse dihydrofolate reductase as well), the inhibitor's pyrimidine ring is inserted 1 Å more deeply into the active site than is the pyrimidine portion of MTX when bound to L. casei dihydrofolate reductase. Moreover, in chicken dihydrofolate reductase, the side chain of Tyr-31 moves 5 Å when TMP binds (see below) suggesting that the structurally homologous phenylalanine in mouse dihydrofolate reductase may also change conformation when TMP binds. A third source of error arises from the misidentification by Birssall et al. (1983) of Phe-34 as a tyrosine residue. Clearly, the exact orientation and chemical identity of Phe-31 and Phe-34 will be of crucial importance in correctly calculating the protein's contribution to TMP's proton chemical shifts and in finally arriving at the correct torsion angles.

Turning next to the individual methoxy groups, we note that torsion angles for both meta substituents (6° and 177°) are essentially identical to those reported for free trimethoxybenzene in solution (Markriyannis and Fesik (1982); and references therein) and differ by less than 20° each from the corresponding torsion angles for TMP bound to E. coli dihydrofolate reductase. The para-methoxy torsional rotations differ by almost 180° for TMP bound to chicken (109°) and to molecule I of E. coli dihydrofolate reductase (−65°) so that in each conformation the methyl groups are equally distant from the face but on opposite sides of the respective benzene rings. Recall that in molecule II of the E. coli dihydrofolate reductase binary complex the orientation of TMP's para-methoxy substituent is influenced by the presence of a bound water molecule that is most probably an artifact of crystallization (see above).

In the chicken dihydrofolate reductase holoenzyme, the side chain of Tyr-31 occupies a position directly underneath that of Glu-38, which is one turn further advanced along helix aB. Upon binding TMP, some conformational relaxation is required in order to relieve what would otherwise be a strong repulsion between one of the meta-methoxy groups of TMP and the side chain of Tyr-31 (e.g. the distance between Ctl of Tyr-31 and C10 of TMP would be less than 2.0 Å in the absence of protein conformational changes). The difference map (Fig. 6) indicates that, upon binding TMP, this tyrosine side chain rotates down into a new position. Here it packs against the polypeptide backbone atoms of residues 26–28 and the side chain of Leu-22, with its hydroxyl group hydrogen bonded via an intervening water molecule (Wat-424) to the backbone carbonyl oxygen of Trp-24. This conformational change is accomplished by rotations about the Ca-C$\beta$ and C$\beta$-Cγ bonds of 105° and 55°, respectively, resulting in an average atomic movement of 5.4 Å for all side chain atoms beyond C$\beta$. In the following paper, we go on to examine a variety of other dihydrofolate reductase inhibitors bound to chicken dihydrofolate reductase. Analyses of those data and comparison with structural results presented in this paper permit identification of the differences in binding that may be important in accounting for TMP's selectivity.

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